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SITE SPECIFIC INTERACTION OF PROTONS LIBERATED FROM PHOTOSYSTEM II OXIDATION WITH A HYDROPHOBIC MEMBRANE COMPONENT OF THE CHLOROPLAST MEMBRANE

L. J. Prochaska and R. A. Dilley,
Department of Biological Sciences, Purdue University
W. Lafayette, IN 47907

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SUMMARY

Chloroplast membranes have been shown previously to undergo a change in radioactive labeling by chemical modification reagents that is dependent on electron transport and protolytic events in Photosystem II. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis has been used to show that a low molecular weight chloroplast polypeptide (7.2 Kilodaltons) undergoes the most change in acetic anhydride labeling upon Photosystem II electron transport. A similar polypeptide has been identified by other workers as a component of the hydrophobic trans-membrane proton channel in chloroplasts. Photosystem I electron transport does not give the change in level of incorporation of acetic anhydride into this membrane protein. These results suggest that protons liberated from Photosystem II interact with a hydrophobic portion of the chloroplast membrane, perhaps with the trans-membrane proton channel.

INTRODUCTION

Chloroplast membranes undergo a change in reactivity with chemical modification reagents such as iodoacetate, diazonium benzenesulfonate, and acetic anhydride that is directly dependent on electron transport and protolytic events in Photosystem II. More diazonium reagent and iodoacetate are incorporated into chloroplasts in the light, (1-5) while less acetic anhydride is bound compared to chloroplasts modified in the dark. (2,5). These changes in incorporation are inhibited by DCMU[1] and are insensitive to uncouplers of photophosphorylation. Chloroplasts treated with hydroxylamine to inhibit water oxidation undergo the changes in labeling pattern when an artificial electron donor that liberates protons upon oxidation (i.e. diphenylcarbazide) is used to restore Photosystem II electron transport, but not when an electron-only Photosystem II electron donor is used [i.e. Iodide (1-3)]. Photosystem I electron and proton accumulation in the presence of DCMU (to block Photosystem II) does not potentiate the change in labeling pattern (1,2,4). These results suggest that protons released by

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[1] Abbreviations: DCMU 2,4-Dichlorophenyl 1,1-Dimethylurea; SDS, Sodium Dodecyl Sulfate; CF_1, Chloroplast Coupling Factor; CF_0, Hydrophobic Component of Chloroplast Coupling Factor; MV, Methylviologen; DCC, N,N' Dicyclohexylcarbodiimide; DCIP, 2,6-Dichlorophenolindolphenol; HEPPS, N-2-Hydroxyethylpiperazine Propane Sulfonic Acid.

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Photosystem II are initially compartmentalized in an intra-membrane domain that is not in communication (equilibrium) with protons translocated by Photosystem I (5). The Photosystem II - dependent change in the incorporation of the chemical modification reagents is interpreted as due to conformational changes in chloroplast thylakoid membrane proteins induced specifically by protons released during water oxidation [or alternate proton donating Photosystem II donor oxidation (1-5)].

In an attempt to clarify the functional role of the Photosystem II-linked conformational change, it is useful to investigate which polypeptides undergo the conformational change. Chloroplast membranes that have been radioactively labeled by acetic anhydride in the light or dark can be dissociated into their component polypeptides by sodium dodecyl sulphate polyacrylamide gel electrophoresis and the amount of radioactive label incorporated into each individual protein band can be determined. The polypeptide(s) having the greatest light-dark difference in acetic anhydride incorporation could represent the peptides most intimately involved in the conformational change.

MATERIALS AND METHODS

Chloroplasts were isolated (9) and chlorophyll concentration was determined as described previously (10). Chloroplasts (0.3 mg/ml) were incubated at 20°C in 50 mM KCl, 50 mM HEPPS NaOH, pH 8.6, 2 mM $MgCl_2$, 0.5 mM MV, 0.5 mM NaN_3, 5 µM valinomycin, and 15 µM Nigericin (2). After preillumination for 30 seconds with two 500 watt actinic light sources, 1 mM[^{3}H] acetic anhydride in methanol (specific activity - 225 mCi/mmol) was added and illumination continued 15 sec. followed by quenching with a large volume of 0.25 M Tris Cl, pH 8.5. Chloroplasts modified in the dark were treated for a similar period of time. The chloroplasts were then washed twice with 0.25 M Tris Cl, pH 8.5, and resuspended in distilled water. Aliquots were assayed for chlorophyll concentration and radioactive label incorporated. The bulk of the derivatized membranes were either delipidated by 80% acetone in preparation for electrophoresis or injected into n-buthanol for isolation of the CF_0 component protein (8). Protein concentration was estimated by the Lowry method (11). The isolation procedure for the CF_0 component protein of Nelson *et al.* (8) was modified by centrifuging the butanol precipitate four times at 20,000 xg for 10 minutes and the diethyl ether precipitation was allowed to continue for 2-3 hours at 4°C. Protein fractions were resuspended in 100 mM Tris Cl, pH 8.5, 0.5 M Urea, 2% SDS, and 2% β-mercaptoethanol and were then boiled from 1-3 minutes prior to electrophoresis. SDS gel electrophoresis as described by Hoober (12) was performed on both the isolated CF_0 protein and the total membrane protein. Protein staining and gel slicing were as described by Smith *et al.* (13) except that 1 ml of H_2O_2 was used to solubilize the gel matrix. The specific activity of incorporation of radioactivity into peptides was related to the amount of peptide by integrating the area under each major band by weighing and summing the counts in the slices under that band. Aldolase, ovalalbumin, chymotrypsinogen, ribonuclease, cytochrome C, and unreduced insulin were used as molecular weight standards. See Table and Figure legends for additional comments on the techniques used to gather and analyze the data.

RESULTS AND DISCUSSION

The radioactive labeling pattern of chloroplast membrane proteins treated

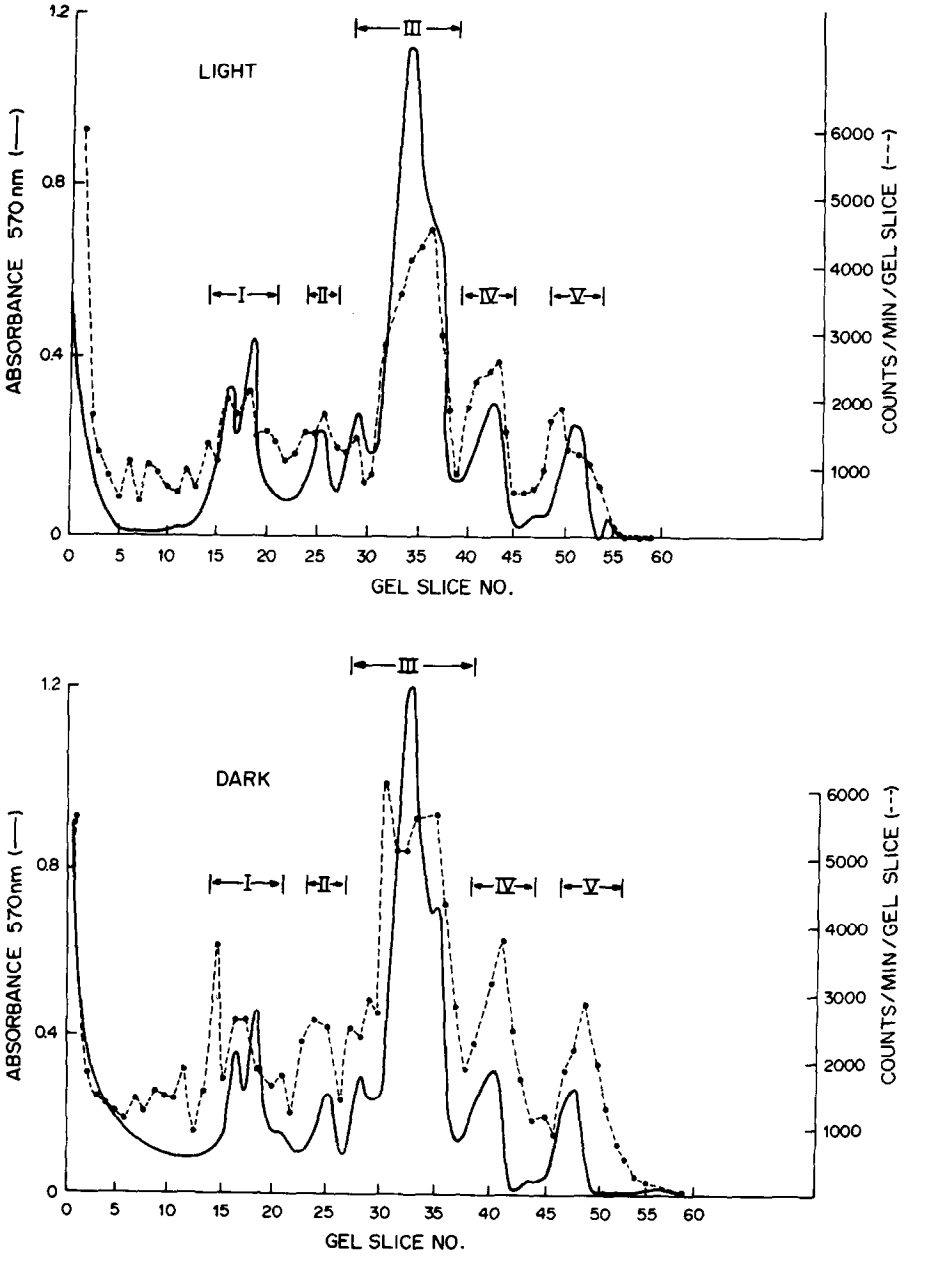

Figure 1. SDS Gel Electrophoresis of Chloroplast Membranes Labeled by Acetic Anhydride in the Light and the Dark. Chloroplast membranes were modified and washed as described in the Materials and Methods section. Delipidated chloroplast membrane proteins were prepared with three 80% acetone washes and resuspended in 100 mM Tris Cl, pH 8.5, 0.5 M urea, 2% SDS, 0.01% EDTA, and 2% β-mercaptoethanol. The gels contained 8% acrylamide and 0.21% N,N Methylenebisacrylamide and were 10 cm in length. The running buffer was 0.5 M urea, 0.2% SDS, 0.01% EDTA, 100 mM Tris Cl, pH 8.5, and 10 mM β-mercaptoacetic acid. Approximately 80 μgms of protein were loaded onto the gels. The gels were run at

with acetic anhydride in the light and dark should give information about which proteins are involved in the Photosystem II dependent membrane conformational change. Figure 1 shows SDS polyacrylamide gels of chloroplasts modified with acetic anhydride either in the light while undergoing water to methyl viologen electron transport or modified in the dark. Polypeptides with molecular weights of 66,57,36,22,13 and 7.2 KD can be observed. Complete resolution of all the membrane peptides was not attempted at this stage (using 8 per cent acrylamide, 10 cm gels), and each of the major bands on the gel shows evidence of minor components as shoulders. The gels were sliced and radioactive label incorporated into each band was determined. (Table I). The most heavily labeled peak in both the light and the dark treated membranes was the 22 KD band [the light-harvesting chlorophyll a, b protein complex (14)]. However, when the counts associated with each protein peak were normalized by area (i.e. specific activity determined), the light-harvesting chlorophyll a, b protein complex was found to have less radioactive label associated with it than the other bands. Since these experiments were designed to identify which proteins underwent a change in radioactive labeling pattern during light activation of chloroplast membranes, the dark-light difference in the specific activity (CPM/unit area) was determined. Comparing Experiment A and B in Table I, it is evident that 59 per cent of the total dark-light difference in acetic anhydride labeling of the membranes occurs in a low molecular weight component (7.2 KD). This polypeptide, though only a minor fraction of the total membrane protein, has a much greater proportion of the total difference in incorporation than any other component. In other experiments the 7.2 KD protein band contained 45 to 60 percent of the difference in labeling.

In seeking further identification of the 7.2 KD polypeptide, we noted that recently, Nelson et al. (8) have isolated a component of CF_0 with an apparent molecular weight of 7,500 that is the site of radioactive DCC binding. This polypeptide is thought to be responsible for both the binding of coupling factor

1.5 mA/tube and were fixed in 20% trichloroacetic acid for two hours. The gels were stained, sliced, and counted as described in the Materials and Methods section. Each protein peak in both the light and dark-modified membrane protein was assigned a numerical classification (I-V) that was consistent for every gel scan. The abscissa for the light and dark-modified membrane protein is not drawn to the same scale. The area under each individual peak was integrated by weighing. The integrated areas and radioactivity associated with each protein peak were uncorrected for overlap. Since similar amounts of the light and dark-modified membranes were loaded onto the gels, the overlap of peaks would be comparable from gel to gel and such overlap is not expected to seriously interfere with the dark-light comparison of integrated peak areas given in Table I. The distribution of membrane protein determined by this method of integration gave consistent reproducibility even at various concentrations of membrane protein loaded onto the gels (40-120 µgms). Staining artifacts such as these described by Fessenden-Raden (26) were not considered. Since the membrane proteins from light and dark-treated chloroplast membranes were prepared and treated at the same time, staining artifacts would not be a problem in the comparison of dark-light differences in each individual protein peak.

(CF_1) to the chloroplast membrane (6,7) and the translocation of protons through the chloroplast membrane. We carried out experiments designed to test whether the peptide we find predominantly labeled is similar to the 7.5 KD CF_0 component isolated by Nelson and colleagues. To our surprise, the protein we label is either identical with, or at least co-purifies with, the CF_0 component. After modification of chloroplasts with acetic anhydride in the light and dark, the protein was isolated by the butanol extraction, ether precipitation procedure from Nelson et al. (8) and electrophoresis on SDS gels was performed. While eight per cent acrylamide gels are not ideal for resolving protein species in the less than 10 KD molecular weight range, the ether precipitate did show a single, symmetric band near 6 KD and several very minor bands of much greater molecular weight. While the isolated 6 KD polypeptide is apparently homogeneous on our SDS gels (not shown) we are currently performing experiments to test this point, using N-terminal amino acid analysis and multiple chromatographic separations, including gradient slab gels. Additional experiments are planned to test whether the putative CF_0 component has the expected proton translocating activity. For the moment we shall leave open the question of the exact identity of the 6 KD polypeptide, but we favor the possibility that we are dealing with the CF_0 component of the energy coupling mechanism.

Table I shows that the isolated 6 KD component from the dark and light treated membranes (Experiments A and B) has the same specific activity as the low molecular weight peak (7.2 KD) from the chloroplast membrane experiment. When various amounts of the isolated 6 KD component polypeptide were loaded onto gels and the radioactivity associated with each integrated area was plotted, a linear relationship resulted (data not shown). Furthermore, the counts associated with the integrated area of the 7.2 KD polypeptide from the chloroplast membrane gels fall directly on the line generated from the isolated 6 KD component. These data provide evidence that the isolated 6 KD polypeptide which we infer may be the CF_0 component and the polypeptide band at 7.2 KD from the chloroplast membrane gels are the same polypeptide.

We suggest that the protein in chloroplasts that undergoes the most change in radioactive labeling during Photosystem II electron transport may be the low molecular weight component of CF_0. Our conclusions may have to be modified if the isolated 6 KD fraction is heterogeneous in its composition. Using the working hypothesis that the labeling difference in light and dark conditions is caused by an interaction between protons released in Photosystem II water oxidation (or alternate proton-releasing Photosystem II donors) with membrane proteins (2,3,5), the above data suggests that the 6 KD protein "communicates" with the Photosystem II proton releasing mechanism.

Table I. The Effects of Photosystem I and II Electron Transport Upon the Incorporation of Acetic Anhydride into Chloroplast Proteins and the Isolated CF_0 Component.

A. LIGHT-TREATED ($H_2O \rightarrow MV$)

Peak	MW(KD) app	CPM	Area of Protein Stain	CPM/Unit Area (Specific Activity)
I	57,66	3424	6.5	530
II	36	1409	4.0	350
III	22	12,922	33.8	380
IV	13	5158	8.8	590
V	7.2	4842	10.5	460
V(isolated)$_6$		-	-	430

B. DARK-TREATED

					Δ Specific Activity Δ (Dark-PS II)	%Δ Specific Activity in Peak
I	57,66	4858	8.7	560	30	8
II	36	1698	4.9	350	0	0
III	22	16,054	39.6	405	25	7
IV	13	6329	9.2	680	90	25
V	7.2	8310	12.4	670	210	59
V(isolated)$_6$		-	-	690	-	-

C. LIGHT-TREATED ($DCIPH_2 \rightarrow MV + DCMU$)

					Δ Specific Activity Δ (Dark-PSI)
I	57,66	4150	8.0	510	50
II	36	1893	5.4	350	0
III	22	18,165	40.2	450	-15
IV	13	8079	10.3	780	-100
V	7.2	8562	12.9	670	0
V(isolated)$_6$		-	-	680	-

Chloroplasts were modified as described in the Materials and Methods section. Additional components of the reaction mixture for the Photosystem I modification were 20 μM DCMU, 50 μM DCIP and 2.5 mM Ascorbate. The rates of electron transport during the modification for the two light treatments were equal. The $DCIPH_2 \rightarrow MV$ electron transport rate was adjusted by lowering the light intensity. SDS gel electrophoresis and isolation of the low molecular weight CF_0 component were described in the Materials and Methods section. Electrophoresis on the CF_0 component and chloroplast membrane proteins was performed concurrently. Both the isolated CF_0 component and the chloroplast membrane gels were corrected for background. The area of protein stain for each peak was integrated by weighing. The Δ Specific Activity column represents the observed change in Specific Activity in each protein peak as a function of treatment. For example, the Δ Specific Activity for the $H_2O \rightarrow MV$ treatment represents the specific activity of the Dark treatment minus the $H_2O \rightarrow MV$ treatment for each individual protein peak.

Only radioactivity associated with the 6 KD band of the isolated CF_0 fraction run on separate SDS gels was used to calculate the specific activity of the isolated band V. Other polypeptides were present in the diethyl ether precipitate. However,

One intriguing aspect of this is that the effect on the 6 KD polypeptide is specific for Photosystem II proton release. Our previous work using thylakoid preparations (without isolating any components) has shown that Photosystem I electron transport, and its attendant proton transport, does not induce the change in radioactive chemical modifier incorporation into the membrane (1,2,4). Consistent with that observation we find that Photosystem I electron transfer (plus DCMU to inhibit Photosystem II) does not induce a change in the level of acetic anhydride incorporation into the 6 KD component (Table I, compare Experiments B and C). The 6 KD component isolated from chloroplasts activated only by Photosystem I has a specific activity equivalent to dark-treated membranes. In these experiments, uncouplers were present to distinguish the conformational changes we are measuring from the uncoupler sensitive conformational changes known to occur in the coupling factor protein (15,16).

The changes in acetic anhydride incorporation into other polypeptides, while considerably less than the changes in the 6 KD component labeling, are, nonetheless, significant. Using a fluorescence probe (fluorescamine) that reacts with primary amino groups, Ellenson et al. (17) reported that three polypeptides of 32,23 and 15 KD molecular weight show less labeling with the probe in the light compared to dark conditions. The three components were suggested to be part of the CF_0 complex (7). Perhaps some of the changes in acetic anhydride labeling observed in our bands III (22 KD) and IV (13 KD) are due to the polypeptides suggested in the work of Ellenson et al. (17). Alternatively, the changes in labeling of these bands could also be due to a massive membrane perturbation induced by the protonation of the 6 KD component, although we observe the changes in the presence of uncouplers.

CONCLUDING REMARKS

Assuming that the 6 KD fraction we isolate contains *only* the CF_0 component of the chloroplast energy coupling mechanisms, we can offer the following comments.

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only the 6 KD (≃ 90% of the protein stain) was analyzed for the specific activity calculation.

The Photosystem II-induced differences in the incorporation of acetic anhydride into chloroplast membrane proteins were observed in seven different chloroplast preparations that were modified in the light or dark (EXP. A and B). The low molecular weight Band (V) accounted for 52 ± 7% of the total dark-light difference in acetic anhydride incorporation into chloroplast membrane polypeptides. The CF_0 component was isolated from only three of these preparations and the specific activity of the isolated CF_0 component from both the light and dark-modified membranes correlated well with the low molecular weight chloroplast membrane polypeptide (V). The absence of the Photosystem I-induced change of acetic anhydride labeling in the CF_0 component was observed twice (EXP. C) in two different preparations.

The above results, along with the previous work from this laboratory on the Photosystem II-linked conformational change (1-5) suggest that protons released by Photosystem II are processed by an intramembrane route or device so as to interact with the CF_0 component via a mechanism which Photosystem I-linked protons cannot activate. The involvement of the CF_0 component, an assumption admitedly requiring further critical testing, implies that the Photosystem II-specific conformational change may be involved in energy transduction in Photosystem II. Previous suggestions from this laboratory (18) as well as recent work from Boyer's (19) and Slater's (20) laboratories have led to suggestions that conformational changes may be involved in energy transduction. Ellenson et al. (17) also interpret their results along similar lines. Photosystem I-linked proton accumulation activates the energy transduction mechanism and the protons thus accumulated undoubtably utilize the CF_0 proton port, but we must conclude that they do so in a manner different from protons derived from Photosystem II oxidations. The nature of the hypothesized intramembrane domains for proton processing from the Photosystem II oxidation site to the CF_0 - CF_1 complex remains to be elucidated.

These results support the concept, first put forth by Williams (21,22), that protons released by the redox reactions of energy transducing membranes, may be utilized in energy requiring reactions via intramembrane processing mechanisms. Moreover, the present data and hypothesis are consistent with (a) the results of Izawa, et al. (23) showing that Photosystem II-linked phosphorylation has a different pH dependence for the ATP per $2e^-$ ratio than Photosystem I phosphorylation; and (b) work in this laboratory (24,25) which showed that protons released in electron transport can be processed into the phosphorylation mechanism, perhaps via an intramembrane route, before being pooled in the inner aqueous space.

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